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Real-Time Multiplex PCR Assay and Melting Curve Analysis for Identifying Diarrheagenic *Escherichia coli*

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A real-time multiplex PCR assay was designed to amplify the virulence genes *eae*, pEAF, *aatA*, *daaC*, *elt*, *est*, *ipaH*, stx_1, and stx_2 for the detection of all diarrheagenic *Escherichia coli* pathotypes. This assay proved to be more sensitive and rapid than a conventional multiplex PCR for diarrheagenic *E. coli* isolates from children with diarrhea.

Diarrheagenic *Escherichia coli* (DEC) strains are an important cause of diarrhea among children in developing countries and are now being recognized as emerging enteropathogens in the developed world (1). Six *E. coli* pathotypes are currently known: enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and Shiga toxin-producing *E. coli* (STEC), and enterohemorrhagic *E. coli* (EHEC). EPEC strains are divided into typical (tEPEC) and atypical EPEC (aEPEC) strains based on the presence or absence of the EAF plasmid, respectively (2).

DEC identification is based on virulence characteristics detected by extremely time-consuming and laborious phenotypic and genotypic assays, such as HEp-2 cell adherence, DNA hybridization, and PCR assays. Since serotyping is the traditional method used in most Brazilian clinical laboratories, DEC strains are probably underdetected.

Here we describe a multiplex real-time PCR that simultaneously detects nine virulence genes associated with the six DEC pathotypes. The previously reported two-reaction multiplex PCR for DEC detection (3) was adapted to a single-reaction real-time multiplex PCR, eliminating the gel electrophoresis step.

Bacterial strains. The prototype strains used as positive controls were EPEC E2348/69, EAEC 042, DAEC C1845, ETEC H10407, EIEC EDL1284, and STEC H30 and 86-84. Eighty additional *E. coli* strains (10 tEPEC, 10 aEPEC, 10 EAEC [$aatA^+$], 10 EAEC [*aatA* mutant], 10 DAEC [$daaC^+$], 10 DAEC [*daaC* mutant], 6 ETEC [*elt*], 2 ETEC [*est*], 1 ETEC [*elt*/*est*], 4 EIEC, 2 STEC [stx_1], 1 STEC [stx_1/stx_2], and 4 EHEC [*eae*, stx_1] strains) representing all *E. coli* pathotypes and obtained from previous clinical studies were also included as controls. These strains were categorized into DEC pathotypes on the basis of DNA probe and/or HEp-2 adherence assays (4–6). Ten commensal *E. coli* strains and other non-DEC enteric pathogens (*Shigella* spp., *Salmonella* spp., and *Yersinia* spp.) were included as negative controls. We also tested 328 recently isolated *E. coli* strains obtained from 97 children with diarrhea living in low-socioeconomic-level communities of Espírito Santo, Brazil.

DNA extraction. DNA was extracted by boiling pooled bacterial colonies (one to five) from each stool sample in 300 μl of sterile distilled water for 10 min and then centrifuging them for 5 min. Two microliters of each sample was used as template DNA.

Primers. The primers were selected to allow the simultaneous detection of nine different virulence genes in a single reaction mixture. The primers for *eae*, pEAF, *aatA* (previously designated pCVD432), *elt*, *est*, *ipaH*, stx_1, and stx_2 have been published elsewhere (7–11). Primers for *daaC* were designed on the basis of the previously published sequence (12) using IDT IciTools (IDT Integrated DNA Technologies, Coralville, IA).

Real-time multiplex PCR. The previously published multiplex PCR (3) was adapted for a real-time multiplex assay by substituting the *bfpA* primers for pEAF primers. The reaction was performed by using the real-time time PCR system Mastercycler ep realplex[4] (Eppendorf North America). Each 40-μl reaction mixture contained 25 μl SYBR GreenER qPCR SuperMix Universal (Life Technologies) and 2 μl of template DNA (Table 1). The reaction mixture was subjected to 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 60 s. After 45 cycles, a melting curve with a ramp speed of 2.0°C/s between 70°C and 95°C was determined with a reading every 0.2°C using SYBR green fluorescence. Melting curves were analyzed by using the Eppendorf Realplex software (version 2.0).

Melting analysis showed nine distinct peaks for all target genes: *est*, 73 ± 0.2°C; *aatA*, 75 ± 0.4°C; *elt*, 78 ± 0.1°C; stx_1, 80 ± 0.3°C; *eae*, 82 ± 0.2°C; pEAF, 84 ± 0.3°C; *ipaH*, 85 ± 0.1°C; *daaC*, 87 ± 0.2°C; and stx_2, 89 ± 0.3°C (Fig. 1). The peaks were clearly distinct from each other, with different average melting temperatures. The proper panel of virulence genes was detected in all prototype DEC strains. Representative strains of each DEC pathotype were analyzed by agarose gel electrophoresis, and the predicted product size was confirmed.

PCR sensitivity. Serially diluted DNA suspensions of each prototype DEC strain were used as the PCR templates for sensitivity evaluation. The lower limit of detection was about 3×10^2 CFU for each target gene.

PCR specificity. All of the 80 representative DEC strains gave the expected signals. In addition, no other crossover signals were observed among non-DEC, *Salmonella* sp., or *Yersinia* sp. strains. As expected, *ipaH* sequences of EIEC and *Shigella* spp. gave similar

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TABLE 1 Primers used for real-time multiplex PCR

Target gene and primer name	Primer sequence (5′–3′)	Concn (μM)	Source or reference
eae			
EAE-S for	ACT GGA CTT CTT ATT RCC GTT CTA TG	0.2	8
EAE-B2 rev	CCT AAA CGG GTA TTA TCA CCA GA		
pEAF			
EP-1 for	GTT CTT GGC GAA CAG GCT TGT C	0.2	8
EP-2 rev	TTA AGC CAG CTA CCA TCC ACC C		
aatA			
EA-1 for	AGG TTT GAT ATT GAT GTC CTT GAG GA	0.1	8
EA-2 rev	TCA GCT AAT AAT GTA TAG AAA TCC GCT GTT		
daaC			
DAA-F	ATT ACG TCA TCC GGG AAG CAC ACA	0.1	This study
DAA-R	GCT TGC TCA TAA AGC CGC AGA CAA		
elt			
LTf	GGC GAC AGA TTA TAC CGT GC	0.2	11
LTr	CGG TCT CTA TAT TCC CTG TT		
est			
STa-F	ATT TTT MTT TCT GTA TTR TCT T	0.4	11
STa-R	CAC CCG GTA CAR GCA GGA TT		
ipaH			
IpaH1	GTT CCT TGA CCG CCT TTC CGA TAC CGT C	0.2	10
IpaH2	GCC GGT CAG CCA CCC TCT GAG AGT AC		
stx_1			
stxA1 598	AGT CGT ACG GGG ATG CAG ATA AAT	0.1	7
stxA1 1015	CCG GAC ACA TAG AAG GAA ACT CAT		
stx_2			
Stx2f	GGC ACT GTC TGA AAC TGC CC	0.1	7
Stx2r	TCG CCA GTT ATC TGA CAT TCT G		

melting peaks. Ninety-seven pools comprising a total of 328 *E. coli* isolates from children with diarrhea were tested for the presence of DEC pathotypes by both the real-time multiplex PCR and the previously described multiplex PCR (3). Both multiplex assays detected 54 DEC strains. In addition, 20 DEC strains were detected by the real-time multiplex PCR (11 EAEC, 3 DAEC, 4 atypical EPEC, and 2 ETEC strains). All discrepant results were further examined by monoplex PCR with individual colonies, confirming the real-time multiplex PCR results. Among positive pools, mixed infections could also be detected by multiplex assays. According to the real-time multiplex PCR, the most common mixed infections were aEPEC plus DAEC (5/17), DAEC plus EIEC (3/17), EAEC plus aEPEC plus DAEC (3/17), and EAEC plus aEPEC plus ETEC (3/17).

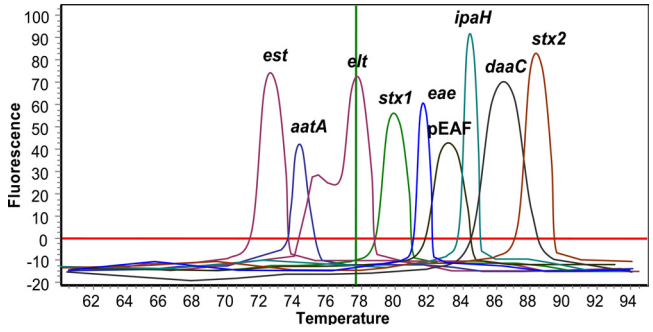

FIG 1 Real-time PCR assay simultaneously detects nine different DEC virulence genes. Data from individual reaction mixtures, each containing DNA from ETEC, EAEC, STEC, EPEC, EIEC, or DAEC strains, are presented in a single graph to show the separation between individual amplicon melting curves.

The real-time multiplex PCR we describe here is unique regarding the number of target genes recognized and has the advantage of detecting all DEC pathotypes and discriminating between tEPEC and aEPEC. The *daaC* and *aatA* genes were chosen for the identification of DAEC and EAEC, respectively, because they are related to the DNA probes usually employed in epidemiological studies (10). However, DAEC (*daaC* mutant) and EAEC (*aatA* mutant) strains are not detected since they lack known virulence markers.

Recently, Guion et al. (13) reported a real-time multiplex PCR designed to detect eight genes for EPEC, EAEC, DAEC, ETEC, EIEC, and STEC identification. However, this assay does not discriminate between tEPEC and aEPEC. In addition, although the

daaD gene used in the assay is conserved, it is not present in the majority DAEC strains.

In conclusion, our real-time multiplex PCR assay proved to be rapid, sensitive, and specific for the simultaneous detection of all DEC pathotypes and could be used in routine diagnostic laboratories. In addition, it might be a useful tool for epidemiological surveillance.

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